FK506 Binding Protein from a Thermophilic Archaeon, *Methanococcus thermolithotrophicus*, Has Chaperone-like Activity in Vitro

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ABSTRACT: The in vitro protein folding activity of an FKBP (FK506 binding protein, abbreviated to MTFK) from a thermophilic archaeon, *Methanococcus thermolithotrophicus*, was investigated. MTFK exhibited FK506 sensitive PPIase (peptidyl prolyl *cis*−*trans* isomerase) activity which accelerated the speed of ribonuclease T1 refolding, which is rate-limited by isomerization of two prolyl peptide bonds. In addition, MTFK suppressed the aggregation of folding intermediates and elevated the final yield of rhodanese refolding. We called this activity of MTFK the chaperone activity. The chaperone activity of MTFK was also inhibited by FK506. Alignment of the amino acid sequences of MTFK with human FKBP12 showed that MTFK has two insertion sequences, consisting of 13 and 44 amino acids, at the N- and C-termini, respectively [Furutani, M., Iida, T., Yamano, S., Kamino, K., and Maruyama, T. (1998) *J. Bacteriol. 180*, 388−394]. To study the relationship between chaperone and PPIase activities of MTFK, mutant MTFKs with deletions of these insertion sequences or with amino acid substitutions were created. Their PPIase and chaperone activities were measured using a synthetic oligopeptide and denatured rhodanese as the substrates, respectively. The far-UV circular dichroism spectra of the wild type and the mutants were also analyzed. The results suggested that (1) the PPIase activity did not correlate with chaperone activity, (2) both insertion sequences were required for MTFK to take a proper conformation, and (3) the insertion sequence (44 amino acids) in the C-terminus was important for the chaperone activity.

Several protein factors are involved in protein folding in vitro and in vivo (*1*). These are peptidyl prolyl *cis*−*trans* isomerase (PPIase),[1] protein disulfide isomerase, and molecular chaperones. PPIases are divided into three structurally unrelated families: the cyclophilin (CyP) family which is inhibited by the immunosuppressant cyclosporine, the FKBP (FK506 binding protein) family which is inhibited by the immunosuppressant FK506 or rapamycin, and the parvulin family which is insensitive to these immunosuppressants (*2*). All, except parvulins, are ubiquitous in eukaryotes and bacteria (*3*) and catalyze the isomerization of Xaa−proline (where Xaa is the preceding amino acid) peptide bonds in both small peptides and polypeptides. Thereby, the PPIases accelerate the speed of protein folding, which is rate-limited by the slow isomerization of Xaa−proline bonds (*4*−*6*).

Apart from human carbonic anhydorase II (HCAII), most of the substrate proteins used so far for the study of protein refolding reactions with PPIase were completely refoldable proteins such as ribonuclease T1 (RNaseT1) (*4*−*8*). There have been some reports suggesting that PPIases have chaperone-like activity in vitro. Porcine kidney 18 kDa CyP (pCyP18) and NK-TR (a human CyP homologue) act not only as PPIase but also as chaperone and are able to protect HCAII from misfolding during refolding in vitro (*7*, *8*). However, Kern et al. (*9*) reported that human 18 kDa CyP (hCyP18), pCyP18, and human FKBP12 (hFKBP12) were able to accelerate the speed of refolding of HCAII, but were unable to protect the folding intermediates from aggregation (*9*). Eukaryotic CyP40 keeps denatured β-galactosidase in a folding-competent, soluble, and proteolysis-resistant state (*10*). The hCyP18, hFKBP12, and bovine serum albumin (BSA) were reported to increase the yield of the antibody Fab fragment during refolding in vitro (*11*). However, this effect was thought to be a result of nonspecific protein−protein interaction, since it was not inhibited by cyclosporine. On the other hand, human FKBP52 (hFKBP52), which is a HSP90 (90 kDa heat shock protein)-associated protein, binds thermally denatured citrate synthase (CS) from porcine heart and protects it from thermal aggregation (*12*). This activity of hFKBP52 was shown to be independent of its PPIase activity.

Recently, we purified a thermostable FK506-sensitive PPIase (MTFK) from a thermophilic methanogen, *Methanococcus thermolithotrophicus*, and cloned the corresponding

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[1] Abbreviations: PPIase, peptidyl prolyl *cis*−*trans* isomerase; CyP, cyclophilin; FKBP, FK506 binding protein; HCAII, human carbonic anhydorase II; RNaseT1, ribonuclease T1; pCyP18, porcine 18 kDa kidney cyclophilin; hCyP18, human 18 kDa cyclophilin; bCyP18, bovine 18 kDa cyclophilin; hFKBP12, human 12 kDa FKBP; MTFK, FK506 binding protein from *M. thermolithotrophicus*; *N*-suc-ALPF-pNA, *N*-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide; FK-W, wild-type MTFK; FK-dB, deletion mutant of MTFK lacking the bulge insertion; FK-dF, deletion mutant of MTFK lacking the flap insertion; FK-dBF, deletion mutant of MTFK lacking both the bulge and flap insertions; FK-β2Fβ3, deletion mutant of MTFK containing the β2, flap, and β3 regions; CD, circular dichroism.

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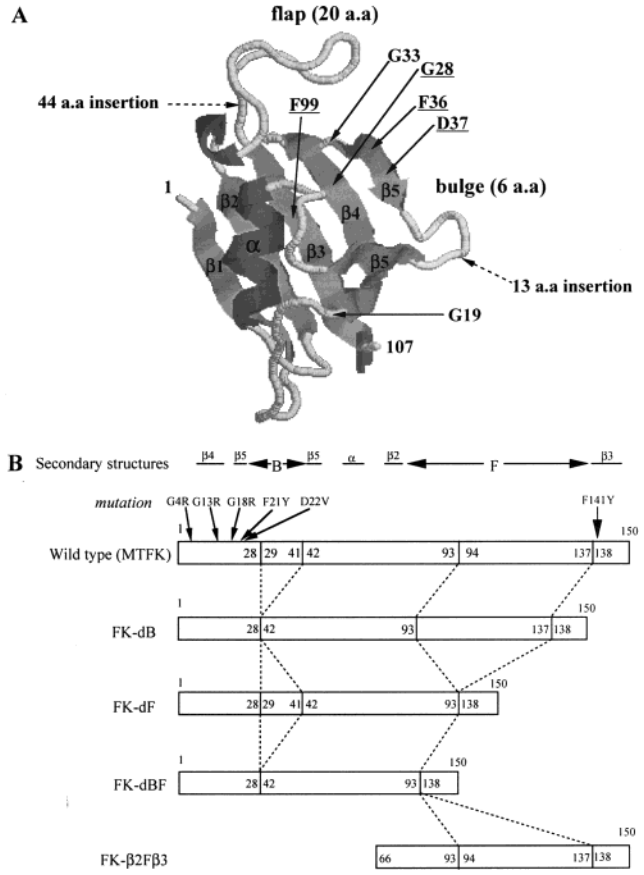

FIGURE 1: Structure of human FKBP12 and the MTFK mutants. (A) Three-dimensional structure of human FKBP12 showing the corresponding sites of MTFK mutants. The structural data (PDB file 1FKK) was obtained from the Protein Data Bank, and the three-dimensional structure was created using Rasmol software. The order of secondary structures of human FKBP12 (107 amino acids) is (N-terminus)−β1−β4−β5−α−β2−β3−(C-terminus). The N- and C-terminal amino acid residues are denoted as 1 and 107, respectively. Between β2 and β3, a surface loop called "flap" exists, and in the middle of β5, an intervening sequence called "bulge" splits β5 in two (*39*). The amino acid sequence corresponding to the β1 strand is missing in MTFK. MTFK has 13- and 44-amino acid insertion sequences in the bulge and flap, respectively (*13*). Deletion mutants of MTFK which lack these insertion sequences were prepared. Arrows show the point mutations in MTFK. The underlined residues (G28, F36, D37, and F99) are involved in the FK506-binding pocket (*30*). (B) Construction of MTFK mutants. The secondary structure of human FKBP12 (except β1) is shown above the wild-type MTFK. The bulge and flap regions of MTFK were deleted to yield mutants that were the same size as human FKBP12. The numbers show the amino acid residues of MTFK. The fifth valine of MTFK reported previously (*13*) was designed as the N-terminal amino acid of the wild-type MTFK in this study. Arrows show the positions of amino acid substitution. Residues G4, R13, R18, F21, D22, and Y141 of MTFK correspond to G19, G28, G33, F36, D37, and F99 of hFKBP12 in panel A. B is bulge and F, flap.

gene (*13*). In comparison with hFKBP12, the amino acid sequence of MTFK has a short (13 amino acids) and a long (44 amino acids) insertion at the regions corresponding to the bulge and flap of hFKBP12 (Figure 1A). While a similar size insertion in the flap region has been found in some FKBP homologues, the insertion in the bulge region is unique to archaeal FKBPs (*13*). FKBP homologues with these insertions, similar to MTFK, were found in the genomes of the thermophilic and hyperthermophilic archaea, *Methanococcus jannaschii*, *Archaeoglobus fulgidus*, and *Methanobacterium thermoautotrophicum* (*14*−*16*). While these FKBPs, including MTFK, are homologous to *Escherichia coli* SlyD and 16 kDa FKBP (ecFKBP16) (*13*, *17*, *18*), the function of this type of PPIase in protein folding is not clear.

In the genomes of *M. jannaschii* (*14*) or *A. fulgidus* (*15*), homologues to group II chaperonin and FKBP have been found. However, these archaea lack genes encoding proteins homologous to other bacterial molecular chaperones such as GroES, DnaK, DnaJ, and GrpE, and a gene for cyclophilin or parvulin. It is likely that group II chaperonin (HSP60) and FKBP are important in protein folding in these thermophilic archaea. Because some PPIases exhibit chaperone-like functions in vitro and in vivo (*8*, *10*, *12*, *19*, *20*), we suspected that MTFK might have chaperone activity in protein folding. In this study, we investigated the protein folding activities of MTFK using RNaseT1 (*5*) and rhodanese (*21*), which are completely and partially refoldable proteins, respectively.

Here we demonstrate that MTFK exhibits not only PPIase activity but also chaperone activity in vitro. The relationship between chaperone and PPIase activities was analyzed by creating mutants with deletions of the bulge and flap insertion sequences, or with amino acid substitutions.

MATERIALS AND METHODS

Materials. Ribonuclease T1 (from *Aspergillus oryzae*, lot no. R7384), rhodanese (from bovine liver, lot no. R1756), recombinant human 12 kDa FKBP (hFKBP12) (lot no. F5398), cyclophilin (from calf thymus, lot no. C7696), and a recombinant *E. coli* Chaperonin60−Chaperonin10 (GroEL−GroES) complex (lot no. C7563) were purchased from Sigma (St. Louis, MO). *N*-Succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide (*N*-suc-ALPF-pNa) was purchased from Peptide Institute, Inc. (Osaka, Japan). FK506 was a gift from Fujisawa Pharmaceutical Co. (Osaka, Japan). It was dissolved in ethanol (EtOH) at a concentration of 2.5 mM and stored at −20 °C. The protein concentration was determined by the Bradford dye binding method (*22*) using a Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard.

Expression of Wild-Type Recombinant MTFK (FK-W) in E. coli. The MTFK gene (450 bp), with *Nco*I and *Bam*HI sites at either end, was amplified by PCR using the primers FKE-F (5′-GGCCATGGTAGATAAAGGAGTTA-3′) and FKE-R (5′-CCGGATCCTTATTCGACAACTTCAATAATT-3′) from a pUC18 plasmid containing the complete MTFK gene isolated from a genome library of *M. thermolithotrophicus* (*13*). In this study, the fifth valine of the MTFK sequence in the previous study (*13*) was designed to be the N-terminal amino acid residue. The FKE-F was designed to contain a *Nco*I site and encodes the N-terminal amino acid sequence (VDKGVK) of MTFK (*13*). The amplified DNA fragment was recovered and digested with *Nco*I and *Bam*HI. It was then ligated into the pET-11d expression vector (Novagen Co., Madison, WI) which had been digested with the same restriction enzymes. This resultant expression vector, pTFK, was introduced into *E. coli* BL21(DE3) cells (Novagen Co.). The transformant was grown in 2× YT broth (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl/L of medium) containing ampicillin (100 μg/mL) at 35 °C. When the OD_{650} reached 0.80−1.0, 1 mM IPTG was added, and then the mixture was incubated for an additional 10 h at 35 °C. The

cells were harvested by centrifugation and disrupted by sonication in 50 mM potassium phosphate buffer (pH 7.5). Expression of the MTFK gene was analyzed by SDS−PAGE. The extract was incubated at 80 °C for 30 min to denature *E. coli* proteins. The supernatant was collected by centrifugation, and ammonium sulfate was added at a final concentration of 1.8 M. After centrifugation, the supernatant was applied to a TSK gel Ether-5PW column (7.5 mm × 7.5 cm; TOSOH Co. Ltd., Tokyo, Japan) equilibrated with 50 mM potassium phosphate (pH 7.0) containing 1.8 M $(NH_4)_2SO_4$. After the proteins were separated using a linear gradient of 1.8 to 0 M $(NH_4)_2SO_4$, the active fraction was collected and dialyzed against 25 mM potassium phosphate buffer (pH 7.5). The dialyzed preparation was applied to a Super Q-5PW column (7.5 mm × 7.5 cm; TOSOH Co. Ltd.) and eluted with a linear gradient of 0 to 0.5 M NaCl. The PPIase active fractions were analyzed by SDS−PAGE. To confirm the N-terminal amino acid sequence of the recombinant wild-type MTFK (FK-W), purified FK-W was electroblotted onto a PVDF membrane after SDS−PAGE and stained with Coomassie brilliant blue R-250. The corresponding band was cut out, and the amino acid sequence was analyzed by automated Edman degradation with a Shimadzu PSQ-2 protein sequencer (Shimadzu Co., Kyoto, Japan). The purified FK-W was dialyzed against 50 mM potassium phosphate buffer (pH 7.5) and used for the following experiments.

Preparation of Deletion Mutants of MTFK. Four deletion mutants of the MTFK gene were constructed. The mutant FK-dB lacks the intervening sequence (A29−Y41) in the bulge region (*13*; Figure 1B). The FK-dF mutant has no intervening sequence (D94−K137) in the flap region. In the FK-dBF mutant, both of the two intervening sequences are deleted. Another deletion mutant, FK-β2Fβ3, expresses only the β2−flap−β3 region (V66−E150) (Figure 1B).

The mutant FK-dB gene was prepared as follows. A DNA fragment (FK-dBN) containing the 5′-half of the FK-dB gene and encoding the region of V1−P43, but lacking the intervening sequence (A29−Y41), was prepared by PCR from pTFK using the primers FKE-F and FKdB-R1 (5′-GGTCTAGAGGTTCTACCTCTTCTATGGAAG-3′). The resultant FK-dBN had *Nco*I and *Xba*I sites at the 5′- and 3′-ends, respectively. The 3′-half DNA fragment, FK-dBC with *Xba*I and *Bam*HI sites at the 5′- and 3′-ends, respectively, was prepared by PCR from pTFK using the primers FKdB-F1 (5′-AACCTCTAGAGTTCGTTGT-3′) and FKE-R. After digestion of the amplified DNA fragments, FK-dBN and FK-dBC, with *Nco*I−*Xba*I and *Xba*I−*Bam*HI, respectively, they were ligated to the *Nco*I−*Bam*HI-digested pET-11d vector. The resultant expression vector was named pTFK-dB.

The FK-dF gene, with *Nco*I and *Bam*HI sites at the 5′- and 3′-ends, respectively, was amplified from pTFK by three successive PCRs using the following primer sets: first, FKE-F and FKdF-R1 (5′-AATTGTAAATACTAAATCTCTTGGTATTTTCTGTATTAACATTTC-3′); second, FKE-F and FKdF-R2 (5′-GACAACTTCAATAATTTTAATTGTAAATACTAAATCTCTTGGTAT-3′); and third, FKE-F and FKdF-R3 (5′-CCGGATCCTTATTCGACAACTTCAATAATTTTAATTGT-3′). After each amplification, 1 μL of the PCR mixture was used as the template for the next PCR in a total volume of 50 μL. The resultant amplified FK-dF gene was digested with *Nco*I−*Bam*HI and ligated to *Nco*I−*Bam*HI-digested pET-11d. This expression vector was called pTFK-dF.

The FK-dBF gene, with *Nco*I and *Bam*HI sites at the 5′- and 3′-ends, respectively, was amplified by three successive PCRs using pTFK-dB as the template with the same primer sets used in the preparation of the FK-dF gene.

The FK-β2Fβ3 gene, with *Nde*I and *Bam*HI sites at the 5′- and 3′-ends, respectively, was amplified from pTFK by PCR using the primers FKf-F (5′-GGCATATGGTCGGGGACGAAAAAACC-3′) and FKE-R. The amplified FK-β2Fβ3 gene was digested with *Nde*I−*Bam*HI and ligated to the *Nde*I−*Bam*HI-digested pET-5a vector (Novagen Co.). The resultant expression vector was named pTFK-β2Fβ3.

After confirmation of the sequences, the expression vectors pTFK-dB, pTFK-dF, pTFK-dBF, and pTFK-β2Fβ3 were introduced into *E. coli* BL21(DE3) cells. The transformants were grown at 35 °C in 2× YT broth containing ampicillin (100 μg/mL). When the OD_{650} of the culture reached 0.80−1.0, 1 mM IPTG was added and the mixture further incubated for 10 h at 35 °C. Expression of the mutant genes was analyzed by SDS−PAGE. To precipitate *E. coli* proteins, the crude extract was incubated at 60 °C for 30 min. All of the mutant proteins, FK-dB, FK-dF, FK-dBF, and FK-β2Fβ3, were purified by a TSKgel Ether-5PW column with a linear gradient of 1.8 to 0 M $(NH_4)_2SO_4$ in 50 mM potassium phosphate buffer (pH 7.0), and then dialyzed against 25 mM potassium phosphate buffer (pH 7.5). The dialyzed preparations were applied to a TSKgel SuperQ-5PW column and eluted with a linear gradient of 0 to 0.5 M NaCl in 25 mM potassium phosphate buffer (pH 7.5). The purified proteins were dialyzed against 50 mM potassium phosphate buffer (pH 7.5).

Preparation of Amino Acid-Substituted Mutants of MTFK. The mutant MTFK genes for amino acid substitutions D22V, F141Y, and F21Y/D22V were prepared by PCR from pTFK. The D22V gene was made by two successive PCRs from pTFK as a template. The primers FKE-F and D22V-R (5′-ACCTCTTCTATGGAAGTGACAAAGACATCTCCACTT-3′) were used in the first PCR, and then the resultant amplified fragment and FKE-R were used as primers in the second PCR. The F141Y gene was produced by three successive PCRs from pTFK using the following three primer sets: first, FKE-F and F141Y-R1 (5′-AATTTTAATTGTATATACTAAATCTTTTCCTGC-3′); second, FKE-F and F141Y-R2(5′-TTATTCGACAACTTCAATAATTTTAATTTTAATTGTATATACTAAATC-3′); and third, FKE-F and FKE-R. The gene for double mutant F21Y/D22V was amplified from pTFK by two successive PCRs using the following two primer sets: first, FKE-F and F21Y/D22V-R (5′-ACCTCTTCTATGGAAGTGACATAGACATCTCCACTTTCA-3′); and second, the amplified fragment of the first PCR and FKE-R. The resultant amplified fragments were digested with *Nco*I−*Bam*HI and then introduced into the *Nco*I−*Bam*HI-digested pET-11d vector. The genes for triple mutants F21Y/D22V/F141Y, G4R/F21Y/D22V, G13R/F21Y/D22V, and G18R/F21Y/D22V were prepared from the pET-11d vector which contained the F21Y/D22V gene. The F21Y/D22V/F141Y gene was made by three successive PCRs using the following three primer sets: first, FKE-F and F141Y-R1; second, FKE-F and F141Y-R2; and third, FKE-F and FKE-R. The G4R/F21Y/D22V gene was ampli-

fied using the primer set consisting of G4R-F (5′-GGCCATGGTAGATAAACGAGTTAAAAT-AAAA-3′) and FKE-R. The G13R/F21Y/D22V gene was amplified by two successive PCRs using the following two primer sets: first, G13R-F1 (5′-GGAGTTAAAATAAAAGTAGACTACATACGTAAACTTGAA-3′) and FKE-R; and second, G13R-F2 (5′-GGCCATGGTAGATAAAGGAGTTAAAATAAAAGTAGAC-3′) and FKE-R. The G18R/F21Y/D22V gene was amplified in the same way using the following three primer sets: first, G18R-F1 (5′-AGTAGACTACATAGGTAAACTTGAAAGTCGAGATGTCTATG-3′) and FKE-R; second, G18R-F2 (5′-GTAGATAAAGGAGTTAAAATAAAAGTAGACTACATAGGTAAACT-3′) and FKE-R; and third, FKE-F and FKE-R. All of the amplified F21Y/D22V/F141Y, G4R/F21Y/D22V, G13R/F21Y/D22V, and G18R/F21Y/D22V genes were digested with *Nco*I−*Bam*HI and then introduced into the *Nco*I−*Bam*HI-digested pET-11d vector. After confirmation of the sequences, all pET-11d vectors harboring the mutated gene were introduced into *E. coli* BL21(DE3) cells. They were expressed in *E. coli* and purified as described for the deletion mutants.

Far-Ultraviolet Circular Dichroism Spectroscopy. Far-UV circular dichroism (CD) spectra were measured with a Jasco J-725 spectrometer (Nippon Bunkou Co., Tokyo, Japan) in a 0.1 cm cell. Samples (3.2−23 μM) were dissolved in 5 mM sodium phosphate (pH 7.4) at 20 °C, and 20 scans between 190 and 250 nm were averaged. The results were normalized to the mean residue ellipticities $[\theta]_{MRW}$. Protein concentrations were estimated using molar adsorption at 280 nm and the amino acid sequences of the wild type and mutants ($\epsilon = 5360$ M^{-1} cm^{-1} for FK-W, FK-dF, and D22V, 2680 M^{-1} cm^{-1} for FK-dB and FK-dBF, 1340 M^{-1} cm^{-1} for FK-β2Fβ3, 6700 M^{-1} cm^{-1} for F141Y, F21Y/D22V, G4R/F21Y/D22V, G13R/F21Y/D22V, and G18R/F21Y/D22V, and 8040 M^{-1} cm^{-1} for F21Y/D22V/F141Y). The FK-dB and FK-dBF mutants were denatured in 5 mM sodium phosphate (pH 7.4) containing 6.0 M guanidine hydrochloride at room temperature overnight. The CD spectra (210−250 nm) of the intact and denatured mutants, FK-dB and FK-dBF, were compared.

PPIase Assay. PPIase activity was determined in a two-step reaction coupled with chymotrypsin using the oligopeptide *N*-suc-ALPF-*p*-nitroanilide as the substrate (*23*). The reaction mixture (final volume, 2.2 mL) contained 17 μM oligopeptides and 0.05−5.0 μM FK-W or the mutant FKBPs in 50 mM potassium phosphate buffer (pH 7.5). The reaction was started by the addition of 50 μL of 1.52 mM chymotrypsin, and the increase in A_{390} that corresponds to the release of pNA was monitored at 25 °C for 3 min with a spectrophotometer (model UV2000, Shimadzu Co.). The catalytic efficiency (k_{cat}/K_m) was calculated from the relationship $K_{obs} = (k_{cat}/K_m)[\text{PPIase}] + K_u$, where K_{obs} and K_u are the first-order rate constants of *p*-nitroanilide release in the presence and absence of the PPIase, respectively, and (k_{cat}/K_m)[PPIase] is the pseudo-first-order rate constant for PPIase-catalyzed isomerization (*23*, *24*). Three different concentrations of the FKBPs were employed. The k_{cat}/K_m value was calculated from the slope of the plot of [PPIase] versus $K_{obs} - K_u$ (K_u was 0.020 s^{-1}).

Refolding of Chemically Denatured RNaseT1 Mediated by Wild-Type MTFK (FK-W). RNaseT1 (50 μM) was denatured by incubation for 2 h in 0.1 M Tris-HCl (pH 7.8) containing 8 M urea and 1.0 mM EDTA at 25 °C. Refolding was started by diluting 55 μL of the denatured RNaseT1 solution with 2.15 mL of refolding buffer 1 [0.1 M Tris-HCl (pH 7.8) and 1 mM EDTA] in the cuvette of a Jasco FP777 model fluorescence spectrophotometer (Nippon Bunkou Co.). The temperature of the cuvette was maintained at 10 °C. Refolding was monitored by the increase in tryptophan fluorescence at 320 nm (5 nm bandwidth) with excitation at 268 nm (3 nm bandwidth).

Refolding of Chemically Denatured Rhodanese Assisted by Wild-Type MTFK (FK-W) and Mutated PPIases. The rhodanese (37.8 μM) was unfolded by incubation for 1 h in 50 mM potassium phosphate buffer (pH 7.8) containing 5 mM *threo*-1,4-dimercapto-2,3-butanediol (DTT) and 6.0 M guanidine hydrochloride (Gdm-HCl) at 25 °C. Refolding was started by 30- or 60-fold dilution with refolding buffer 2 [10 mM DTT, 50 mM sodium thiosulfate, and 50 mM potassium phosphate (pH 7.8)] in the presence of 0−5.0 μM FK-W at 35 °C. BSA and *E. coli* GroE (GroEL−GroES complex) were used as the negative and positive controls, respectively. After incubation for 45 min, rhodanese activity in the refolding mixtures was measured according to the method of Horowitz (*21*). To investigate the effect of FK-W on the aggregation of the folding intermediate during the refolding of rhodanese, 6 M Gdm-HCl-denatured rhodanese was diluted 100-fold with refolding buffer 2 containing 1 or 2 μM FK-W at 35 °C. The time courses of aggregation of rhodanese were monitored by light scattering at 320 nm with a Jasco FP-777 fluorescence spectrophotometer for 20 min. The refolding of denatured rhodanese at 25 or 35 °C, in the presence of 5 μM recombinant hFKBP12, calf thymus cyclophilin, or FK-W, was initiated by 60-fold dilution as described above. All reaction mixtures for the rhodanese refolding contained 1% EtOH because the ethanol solution of FK506 was used for inhibition studies. To study the refoldings of rhodanese using MTFK mutants, denatured rhodanese (37.8 μM) was 60-fold diluted in the presence of 5.0 μM mutants or FK-W, and rhodanese activities were measured after incubation for 60 min at 35 °C.

RESULTS

Expression of MTFK and Mutant Genes in E. coli. Expressed recombinant wild-type MTFK (FK-W, 17 kDa) was detected by SDS−PAGE analysis (Figure 2A, lane 1). It was purified to homogeneity by heat treatment and hydrophobic interaction chromatography with a TSKgel Ether-5PW column (Figure 2B, lane 1). The sequence of the N-terminus of recombinant FK-W was determined to be MVDKGVKIKVD. The amino acid residues, except the first methionine, were identical to that of natural MTFK (nMTFK) purified from *M. thermolithotrophicus* (*13*). The catalytic efficiency (k_{cat}/K_m) of the PPIase activity of FK-W was 0.96−1.0 μM^{-1} s^{-1} for *N*-suc-ALPF-pNA at 25 °C. The FK-W was sensitive to FK506 with an IC_{50} value of 200 nM (data not shown). These characteristics of FK-W were similar to those of nMTFK (*13*).

Mutant genes were also expressed in *E. coli* using the same T7 promoter system. Bands corresponding to the four deletion mutants, FK-dB, FK-dF, FK-dBF, and FK-β2Fβ3, were detected in crude extracts of *E. coli* by SDS−PAGE

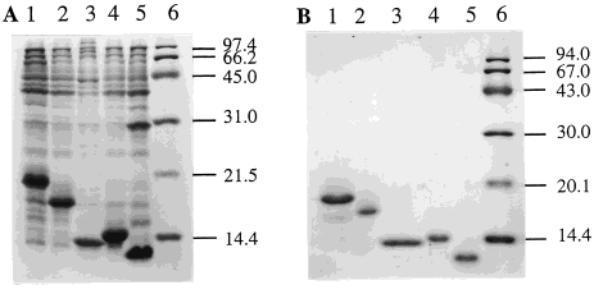

FIGURE 2: SDS−PAGE analysis of the expression of wild-type MTFK and deletion mutants in *E. coli* and their purification. (A) Lane 1, crude extract of the *E. coli* BL21(DE3) strain harboring pTFK induced by 1 mM IPTG. Lane 2, crude extract of the *E. coli* BL21(DE3) strain harboring pTFK-dB induced by 1 mM IPTG. Lane 3, crude extract of the *E. coli* BL21(DE3) strain harboring pTFK-dF induced by 1 mM IPTG. Lane 4, crude extract of the *E. coli* BL21(DE3) strain harboring pTFK-dBF induced by 1 mM IPTG. Lane 5, crude extract of the *E. coli* BL21(DE3) strain harboring pTFK-β2Fβ3 induced by 1 mM IPTG. Lane 6, molecular mass standards. (B) Lane 1, purified wild-type MTFK (FK-W). Lane 2, purified FK-dB. Lane 3, purified FK-dF. Lane 4, purified FK-dBF. Lane 5, purified FK-β2Fβ3. Lane 6, molecular mass standards.

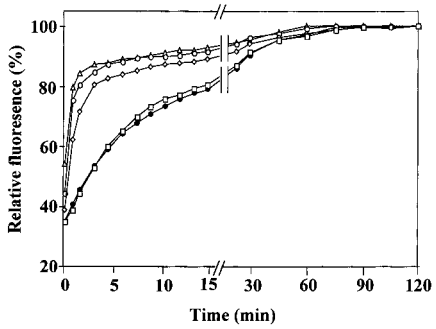

FIGURE 3: Refolding of ribonuclease T1 assisted by wild-type MTFK (FK-W). The 8 M urea-denatured ribonuclease T1 (50 μM) was diluted 40-fold with the refolding buffer [0.1 M Tris-HCl (pH 7.8) and 1 mM EDTA] containing 0−12.5 μM FK-W. The reactions were conducted at 10 °C. The final concentration of ribonuclease T1 was 1.25 μM. The refolding buffer contained (□) 0 μM FK-W, (◇) 1.7 μM FK-W, (○) 6.25 μM FK-W, (△) 12.5 μM FK-W, and (●) 6.25 μM FK-W and 20 μM FK506.

analysis (Figure 2A, lanes 2−5). They were purified to homogeneity (Figure 2B, lanes 2−5). The mutant genes for amino acid substitutions were also expressed in *E. coli*, and purified to homogeneity (data not shown).

Effect of Wild-Type MTFK (FK-W) on the Refolding of RNaseT1. The refolding rate of urea-denatured RNaseT1 was accelerated by FK-W in a dose-dependent fashion (0−12.5 μM) (Figure 3). This effect of FK-W was completely inhibited by 20 μM FK506. This refolding reaction proceeded in a biphasic fashion (*5*; Figure 3). The fluorescence was recovered quickly to 80−85% of that of the intact enzyme within 5 min, and then was recovered gradually to 100% over the course of 60 min.

Effect of Wild-Type MTFK (FK-W) on the Refolding of Rhodanese. To investigate the effect of FK-W on the refolding of rhodanese, rhodanese activity was assayed after incubation in the presence of FK-W, BSA, or the *E. coli* chaperonin complex, GroEL−GroES (Figure 4). Refolding was started by 30- or 60-fold dilution. After dilution, FK-W enhanced the recovery of active rhodanese, like GroEL−GroES with ATP. After 60-fold dilution, the denatured

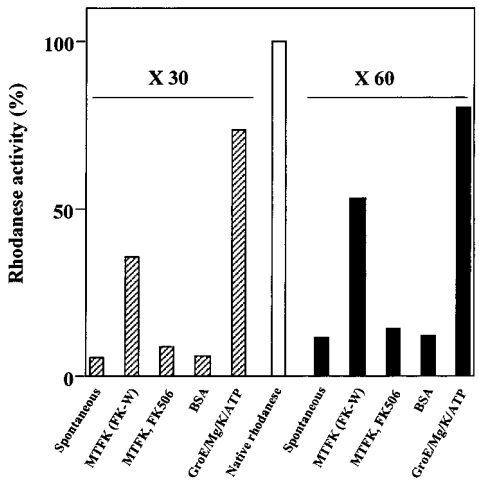

FIGURE 4: Reactivation of rhodanese in the presence and absence of wild-type MTFK (FK-W) and *E. coli* chaperonin. The 6 M guanidine hydrochloride-denatured rhodanese was injected into the dilution buffer [10 mM DTT, 50 mM sodium thiosulfate, and 50 mM potassium phosphate (pH 7.8)]. The cross-hatched columns (left side) show the rhodanese activity 45 min after 30-fold dilution, and the black columns (right side) show the activity 45 min after 60-fold dilution. The central white column shows the activity of nondenatured rhodanese (100%). The dilution buffer of both 30- and 60-fold dilution experiments contained 5 μM FK-W, 5 μM BSA, or 0.5 μM GroE complex. In the case of the reactivation in the presence of the GroE complex, the dilution buffer contained 1 mM $MgCl_2$, 10 mM KCl, and 1 mM ATP. The final concentration of rhodanese was 1.26 and 0.63 μM in the 30- and 60-fold dilution experiments, respectively.

rhodanese was reactivated to 54 and 80% of the original activity, by FK-W and GroEL−GroES with ATP, respectively, while spontaneously, the recovery was 12%. BSA, which was used as a negative control, had little effect on the refolding of rhodanese. The effect of FK-W was strongly inhibited by FK506 (Figure 4). FK506 alone had no effect on the spontaneous refolding of rhodanese (data not shown). To investigate the effect of FK-W on the process of rhodanese refolding, reactions were monitored for 140 min (Figure 5A). While the spontaneous recovery of rhodanese reached a plateau at 14% within 50 min, it was elevated to 31 and 63% by the addition of 1 and 5 μM FK-W, respectively. This effect of FK-W (5 μM) was inhibited by FK506 (0, 2, and 20 μM) in a dose-dependent fashion. However, the inhibitory effect of FK506 on the refolding by FK-W was partial, and the final recovery of the rhodanese activity was approximately 25% in the presence of 20 μM FK506 (Figure 5A).

The stoichiometry of the MTFK−rhodanese interaction was also investigated (Figure 5B). The saturation effect was observed at 15−20 μM MTFK (the MTFK/rhodanese ratio was 20−30), and at 15 μM MTFK, the final yield of refolding reached 90% of that of the native rhodanese. During rhodanese refolding, the aggregation of folding intermediates was investigated (Figure 6). In the absence of FK-W, rhodanese formed aggregations after dilution, and FK-W was able to suppress this aggregation in a dose-dependent fashion. Complete suppression of aggregation was observed in the presence of 2 μM FK-W for 20 min. This effect of FK-W was also inhibited by FK506 in a dose-dependent fashion. FK506 alone had no effect on the aggregation of rhodanese.

Effect of Human 12 kDa FKBP (hFKBP12) and Bovine 18 kDa Cyclophilin (bCyP18) on the Refolding of Rhodanese.

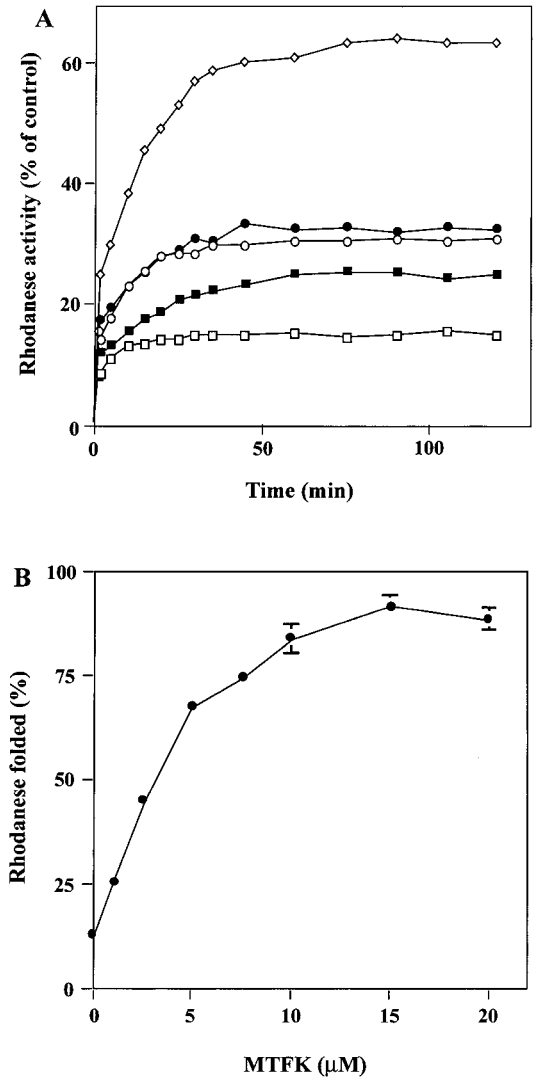

FIGURE 5: Reactivation of rhodanese by wild-type MTFK (FK-W). (A) Time course analysis of rhodanese refolding assisted by FK-W. The 6 M guanidine hydrochloride-denatured rhodanese (37.8 μM) was diluted 60-fold with refolding buffer [10 mM DTT, 50 mM sodium thiosulfate, and 50 mM potassium phosphate (pH 7.8)] and incubated at 35 °C. The final concentration of rhodanese was 0.63 μM. The refolding buffer contained (□) 0 μM FK-W, (○) 1 μM FK-W, (◇) 5 μM FK-W, (●) 5 μM FK-W and 2 μM FK506, and (■) 5 μM FK-W and 20 μM FK506. (B) The stoichiometry of the MTFK−rhodanese interaction. The denaturation and refolding were performed under the same condition described for panel A. After reaction for 60 min, the rhodanese activity of each reaction mixture was assayed. The plots represented are the average of three experiments. Error bars indicate the range of variability between experiments.

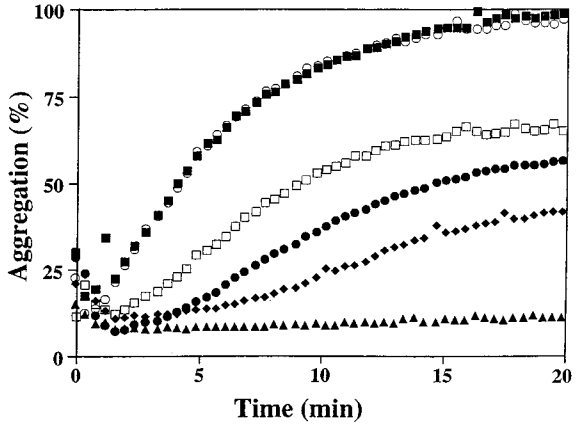

FIGURE 6: Suppression of rhodanese aggregation by wild-type MTFK (FK-W) during refolding. The 6 M guanidine hydrochloride-denatured rhodanese (37.8 μM) was diluted 100-fold with the refolding buffer [10 mM DTT, 50 mM sodium thiosulfate, and 50 mM potassium phosphate (pH 7.8)] containing FK-W. The reactions were conducted at 35 °C. Aggregation was monitored by light scattering at 320 nm. The refolding buffer contained (■) 0 μM FK-W, (●) 0.5 μM FK-W, (▲) 2.0 μM FK-W, (◆) 2.0 μM FK-W and 2.0 μM FK506, (□) 2.0 μM FK-W and 20 μM FK506, and (○) 20 μM FK506.

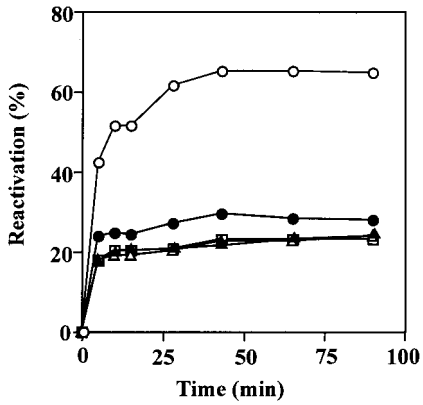

FIGURE 7: Time course of reactivation of rhodanese in the presence of wild-type MTFK (FK-W) and other PPIases. The 6 M guanidine hydrochloride-denatured rhodanese was diluted 60-fold with the refolding buffer [10 mM DTT, 50 mM sodium thiosulfate, and 50 mM potassium phosphate (pH 7.8)] containing PPIases. The reactions were conducted at 25 °C. The refolding buffer contained (□) 0 μM PPIase, (○) 5 μM FK-W, (●) 5 μM FK-W and 20 μM FK506, (▲) 5 μM human recombinant FKBP12, and (△) 5 μM bovine 18 kDa cyclophilin.

To investigate the effect of PPIases other than MTFK on the refolding of rhodanese, hFKBP12 and bCyP18 were used. Neither hFKBP12 nor bCyP18 affected the refolding of rhodanese at 25 (Figure 7) or 35 °C (data not shown). Only MTFK exhibited chaperone activity at both temperatures.

PPIase and Chaperone Activities of Wild-Type MTFK (FK-W) and Deletion Mutants. The catalytic efficiency (k_{cat}/K_m) of the PPIase activity of FK-W was 0.96 μM^{-1} s^{-1}. The yield of denatured rhodanese refolding by FK-W was 64%. The k_{cat}/K_m value of the PPIase activity of FK-dB, a mutant which lacks the insertion in the bulge region, was only 0.004 μM^{-1} s^{-1} (0.4% of that of FK-W). However, FK-dB refolded the Gdm-HCl-denatured rhodanese to 45% of that of the native form (62% of the yield with FK-W) (Table 1). The catalytic efficiency of PPIase activity of the double deletion mutant, FK-dBF, which lacks both bulge and flap insertions, was 0.006 μM^{-1} s^{-1} (0.6% of that of FK-W), which was higher than that of FK-dB. However, FK-dBF did not exhibit chaperone activity (Table 1). The PPIase activity of the FK-dF mutant lacking flap insertion was undetectable even at a concentration of 5 μM. It did not exhibit detectable chaperone activity. The FK-β2Fβ3 peptide containing the flap region of FK-W (Figure 1B) did not exhibit detectable chaperone activity. No chaperone activity was observed in refolding experiments using the refolding buffer containing both FK-β2Fβ3 and FK-dF. This indicated that FK-β2Fβ3 had no complementary effect with the FK-dF.

PPIase Activity and Chaperone Activity of the Amino Acid Substitution Mutants. While the catalytic efficiency of the PPIase activity of the amino acid substitution mutant D22V

Table 1: Deletion Analysis of FKBP from *M. thermolithotrophicus*[a]

MTFKs	PPIase activity [k_{cat}/K_m (mM^{-1} s^{-1})]	chaperone activity [refolded rhodanese (%)][e]
wild type (FK-W)	0.96 (100%)	64.2 ± 2.8 (100%)
FK-W and FK506	0 (0%)	30.0 ± 0.7 (16%)
none[b]	0	14.1 ± 0.4 (0%)
FK-dB	0.004 (0.4%)	45.3 ± 1.7 (62%)
FK-dF	undetectable[c] (0%)	14.5 ± 0.4 (0.8%)
FK-dBF	0.006 (0.6%)	15.4 ± 0.7 (2.6%)
FK-β2Fβ3	ND[d]	14.0 ± 0.5 (−0.2%)
FK-β2Fβ3 and FK-dF	ND[d]	14.9 ± 0.2 (1.6%)

[a] PPIase activity was assayed at 25 °C in a chymotrypsin-coupled assay (Materials and Methods) in 50 mM potassium phosphate buffer (pH 7.5). For the PPIase assay, three different concentrations of each PPIase were employed: for FK-W, 25, 40, and 50 nM; for FK-dB, 2.0, 3.0, and 4.0 μM; and for FK-dBF, 2.0, 3.5, and 5.0 μM. For chaperone activity, the denatured rhodanese (37.8 μM) was diluted 60-fold in the presence of 5.0 μM mutants or FK-W in 50 mM potassium phosphate buffer (pH 7.8) containing 10 mM DTT and 50 mM sodium thiosulfate at 35 °C. [b] Spontaneous refolding of rhodanese without PPIase. [c] No PPIase activity was detected with 5 μM mutant. [d] Not determined. [e] Recovered rhodanese activity after refolding for 60 min in the presence of FKBPs. Values represent means ± the standard deviation ($n = 3$).

Table 2: Amino Acid Substitution Analysis of FKBP from *M. thermolithotrophicus*[a]

MTFKs	PPIase activity [k_{cat}/K_m (mM^{-1} s^{-1})]	chaperone activity (% of rhodanese activity)[d]
wild type (FK-W)	1.0 (100%)	66.7 ± 3.3 (100%)
D22V	0.69 (69%)	55.6 ± 2.6 (78%)
F141Y	0.072 (7.2%)	20.6 ± 1.6 (8.3%)
F21Y/D22V/F141Y	undetectable[c] (0%)	15.3 ± 1.4 (−2.2%)
F21Y/D22V	0.013 (1.3%)	67.4 ± 4.1 (101%)
G4R/F21Y/D22V	0.009 (1.0%)	62.7 ± 3.3 (92%)
G13R/F21Y/D22V	0.006 (0.6%)	32.4 ± 2.6 (32%)
G18R/F21Y/D22V	0.014 (1.4%)	66.8 ± 2.5 (100%)
none[b]	0	16.4 ± 1.5 (0%)

[a] PPIase activity was assayed at 25 °C in a chymotrypsin-coupled assay (Materials and Methods) in 50 mM potassium phosphate buffer (pH 7.5). For the PPIase assay, three different concentrations of each PPIase were employed: for FK-W, 25, 40, and 50 nM; for FK-dB, 2.0, 3.0, and 4.0 μM; for D22V, 35, 50, and 75 nM; for F141Y, 0.3, 0.5, and 0.7 μM; for F21Y/D22V, 1.5, 2.0, and 2.5 μM; for G4R/F21Y/D22V, 1.0, 2.0, and 3.0 μM; for G13R/F21Y/D22V, 1.8, 2.5, and 5.0 μM; and for G18R/F21Y/D22V, 1.8, 2.5, and 4.6 μM. For chaperone activity, the denatured rhodanese (37.8 μM) was diluted 60-fold in the presence of 5.0 μM mutants or FK-W in 50 mM potassium phosphate buffer (pH 7.8) containing 10 mM DTT and 50 mM sodium thiosulfate at 35 °C. [b] Spontaneous refolding of rhodanese without PPIase. [c] No PPIase activity was detected with 5 μM mutant. [d] Recovered rhodanese activity after refolding for 60 min in the presence of FKBPs. Values represent means ± the standard deviation ($n = 3$).

was reduced to 0.69 μM^{-1} s^{-1} (69% of that of FK-W), this mutant refolded rhodanese to 56% of the original activity (78% of that of FK-W) (Table 2). The catalytic efficiency of PPIase activity of the double mutant F21Y/D22V was reduced to 0.013 μM^{-1} s^{-1} (1.3% of that of FK-W) (Table 2). This double mutant refolded rhodanese to 67% of the original activity (101% of that of FK-W). The F141Y mutant exhibited a higher catalytic efficiency (0.072 μM^{-1} s^{-1}, 7.2% of that of FK-W) than that of the F21Y/D22V double mutant. However, this mutant exhibited little chaperone activity (8.3% of that of FK-W). The triple mutant F21Y/D22V/F141Y exhibited neither PPIase nor chaperone activity. The PPIase activities of other triple mutants, G4R/F21Y/D22V, G13R/F21Y/D22V, and G18R/F21Y/D22V, were 0.009, 0.006, and 0.014 μM^{-1} s^{-1}, respectively (Table 2). While the PPIase activities of these triple mutants were similar, G4R/F21Y/D22V and G18R/F21Y/D22V refolded rhodanese to 63% (92% of that of FK-W) and 67% (100% of that of FK-W) of the original rhodanese activity, respectively, but G13R/F21Y/D22V refolded it only to 32% of the original (32% of that of FK-W).

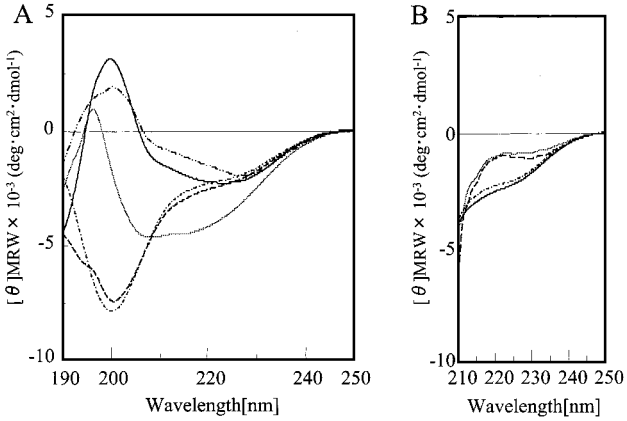

FIGURE 8: (A) Far-UV CD spectra of FK-W and deletion mutants. The spectra were obtained in 5 mM sodium phosphate buffer (pH 7.4) at 20 °C. The data were normalized to $[\theta]_{MRW}$: (—) FK-W, (− − −) FK-dB, (−••−••−) FK-dF, (−•−•−) FK-dBF, and (•••) FK-β2Fβ3. (B) The spectra of intact and denatured mutants, FK-dB and FK-dBF. FK-dB and FK-dBF were denatured in 5 mM sodium phosphate buffer (pH 7.4) containing 6 M guanidine hydrochloride at room temperature overnight. The spectra were obtained in the same buffer at 20 °C. The data were normalized to $[\theta]_{MRW}$: (—) intact FK-dB, (− − −) denatured FK-dB, (−••−••−) intact FK-dBF, and (•••) denatured FK-dBF.

Far-UV CD Spectroscopy. To estimate the secondary structure of the mutant MTFKs, their far-UV CD spectra were studied (Figure 8A). The data showed that (1) the spectra of FK-dF and FK-β2Fβ3 were intermediate between intact and randomized, suggesting their secondary structures were partially randomized, and (2) FK-dB and FK-dBF exhibited a deep minimum at 200 nm, indicating that their secondary structures were significantly randomized. These suggest that the both bulge (13 amino acids) and flap (44 amino acids) insertions are required for correct folding. However, the 210−250 nm region of the spectra of intact FK-dB and FK-dBF were different from their spectra measured in 6 M guanidine hydrochloride (Figure 8B). This suggested that some of the secondary structures of FK-W remained in the mutants, FK-dB and FK-dBF.

The CD spectra of the amino acid substitution mutants F141Y, D22V, F21Y/D22V, and G4R/F21Y/D22V were similar to that of FK-W (Figure 9A,B). This indicated that three-dimensional structures of these mutants remained intact. The spectrum of F21Y/D22V/F141Y exhibited a deep minimum at 200 nm, indicating that its secondary structure was significantly randomized. The CD spectra of G18R/F21Y/D22V and G13R/F21Y/D22V were intermediate between intact and randomized structure, suggesting their secondary structures were partially randomized.

DISCUSSION

PPIases are abundant and widely distributed in organisms from bacteria to mammals (*3*). Both FKBP and cyclophilin

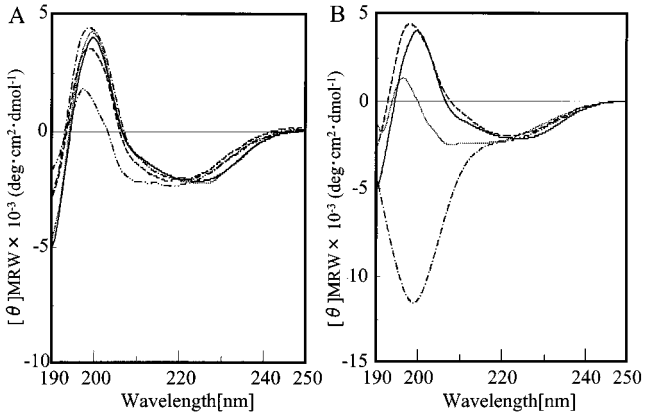

FIGURE 9: Far-UV spectra of wild-type MTFK (FK-W) and amino acid substitution mutants. The spectra were obtained in 5 mM sodium phosphate buffer (pH 7.4) at 20 °C. (A) CD spectra of the wild type and mutants possessing chaperone activity: (—) FK-W, (– – –) G4R/F21Y/D22V, (–·–·–) F21Y/D22V, (–··–··–) G18R/F21Y/D22V, and (···) D22V. (B) CD spectra of mutants with reduced chaperone activity: (—) FK-W, (– – –) F141Y, (–·–·–) F21Y/D22V/F141Y, and (···) G13R/F21Y/D22V.

types of PPIases have been shown to accelerate the refolding rate of proteins in which peptidyl prolyl isomerization is rate-limiting (*4*−*6*, *9*, *25*). However, the physiological functions of PPIases in cells are not well understood. PPIases are distinct from molecular chaperones; the latter bind to folding intermediates and release them often in an energy (ATP)-dependent manner, and suppress aggregation of folding intermediates. However, there have been some reports suggesting in vitro and in vivo chaperone-like functions of PPIases (*8*, *10*, *12*, *19*, *20*).

The RNaseT1 and rhodanese have been employed in studying the PPIase and chaperone functions of MTFK in protein folding, respectively. Because the isomerizations of two peptidyl prolyl bonds (Tyr38−Pro39 and Ser54−Pro55) of RNaseT1 are rate-limiting steps in its folding, this model protein is used to investigate the function of PPIase in protein folding (*5*, *26*). Because the level of spontaneous recovery of chemically denatured bovine liver rhodanese is low (less than 20% of the native one), rhodanese is useful as a model substrate protein in investigating chaperone activity (*21*). *E. coli* GroE binds to folding intermediates of rhodanese and releases them in an ATP-dependent manner (*21*, *27*). GroE protects unfolded proteins from aggregation during refolding and elevates the final yield of active molecules. Bovine liver rhodanese (Swiss-Prot accession number P00586) has 17 peptidyl prolyl bonds. We speculated that if the rate-limited isomerization of some of these bonds is involved in aggregation in the rhodanese refolding, PPIases might suppress aggregation and exhibit chaperone activity.

MTFK exhibited FK506-sensitive acceleration of the folding rate of RNaseT1 (Figure 3). However, 2 μM *E. coli* cyclophilin (ecCyP) completed the biphasic refolding of RNaseT1 within 5 min under the same condition (*5*). The difference in refolding activities of MTFK and ecCyP in RNaseT1 refolding can be explained by the difference in their catalytic efficiencies. The catalytic efficiency (k_{cat}/K_m) of ecCyP against *N*-suc-ALPF-pNA was reported to be 23 μM^{-1} s^{-1} (*28*), but that of MTFK was 1.0 μM^{-1} s^{-1}.

Using rhodanese, we showed that MTFK suppressed its aggregation and elevated the final yield of active rhodanese (Figures 4−6). The denatured rhodanese (0.63 μM) was refolded to approximately 90% of the activity of the native protein in the presence of 15 μM MTFK (Figure 5B). We named this activity the chaperone activity of MTFK. While MTFK-dependent RNaseT1 refolding was completely inhibited by 20 μM FK506 (Figure 3), some chaperone activity remained in the presence of 20 μM FK506 during rhodanese refolding (Figures 5A and 6). The important residues for chaperone activity may be different from those for PPIase activity in RNaseT1 refolding. It is not clear whether the chaperone activity of MTFK is a result of only its PPIase activity.

If the chaperone activity of MTFK in rhodanese refolding is dependent on PPIase activity, other PPIases may have chaperone activity. However, neither hFKBP12 nor bCyP18 exhibited chaperone activity at 25 (Figure 7) or 35 °C (data not shown). The k_{cat}/K_m values of bCyP18 and hFKBP12 against *N*-suc-ALPF-pNA are 2.7 and 0.64−2.2 μM^{-1} s^{-1}, respectively (*3*, *24*). These suggest that the PPIase activity of MTFK is insufficient to explain its chaperone activity.

While the amino acid residues which were supposed to be important for PPIase activity were present in all three deletion mutants, FK-dB, FK-dF, and FK-dBF, their PPIase activities were dramatically reduced (Table 1). The CD spectra of mutant proteins showed that (1) the secondary structures of FK-dF and FK-β2Fβ3 were partially randomized and (2) the secondary structures of FK-dB and FK-dBF were severely randomized (Figure 8A). These suggested that both bulge and flap insertions were important in MTFK assuming a proper conformation for exerting PPIase activity.

Unlike FK-dF and FK-dBF, which did not exhibit chaperone activity, FK-dB retained 62% of the activity of FK-W (Table 1). This suggested that the flap insertion is required for chaperone activity in MTFK, and that the contribution of PPIase activity to chaperone activity was low. The CD spectra of both FK-dB and FK-dBF indicated that their secondary structures were significantly randomized. Spectral differences between the intact and denatured mutants suggested that some secondary structures remained intact in these mutants (Figure 8B). The remaining secondary structures in FK-dB seemed to be responsible for the chaperone activity.

The PPIase activities of the D37V and F36Y mutants of hFKBP12 were reported to retain less than 10 and 0.1% of the wild-type activity, respectively (*29*). The corresponding residues, F21 and D22 (Figure 1B), in MTFK were replaced with Y and V, respectively. While the double mutant F21Y/D22V exhibited 1.3% of the PPIase activity of FK-W, it exhibited chaperone activity comparable to that of the wild type (101% of that of FK-W). This indicated that F21 and D22 were important for PPIase activity, but not for chaperone activity. Three glycine residues (G4, G13, and G18) of the MTFK are highly conserved in FKBPs (*13*). The glycine residue G13 in MTFK which corresponds to G28 of hFKBP12 is involved in the FK506-binding pocket (*30*). These residues seem to be important for PPIase activity, and these residues of the F21Y/D22V mutant were substituted with arginine in the triple mutants. While all of these triple mutants exhibited weak PPIase activity, all but one (G13R/F21Y/D22V) exhibited chaperone activity comparable to that of the wild type (Table 2). While F141Y exhibited PPIase activity higher than those of F21Y/D22V, G4R/F21Y/D22V, and G18R/F21Y/D22V, this single mutant exhibited little

chaperone activity. This indicates that F141 and G13 may be important not only for PPIase but also for chaperone activity.

No mutant that had intact chaperone activity without PPIase activity was obtained. However, the mutants F21Y/D22V and G4R/F21Y/D22V, which had CD spectra similar to that of FK-W (Figure 9A), had almost intact chaperone activity but little PPIase activity (Table 2). The F141Y mutant, which had a CD spectra similar to that of FK-W (Figure 9B), had higher PPIase activity than those of F21Y/D22V and G4R/F21Y/D22V, but little chaperone activity (Table 2). These mutation analyses of MTFK suggest that chaperone activity is independent of PPIase activity.

While cyclophilin was found in a halophilic archaeon, *Halobacterium cutirubrum* (*31*, *32*), no cyclophilin has yet been found in thermophilic and hyperthermophilic archaea. In archaeal genomes, FKBP homologues with two insertion sequences similar to MTFK are the only PPIase found so far, and other FKBPs such as trigger factor have not been found (*14*−*16*, *33*). MTFK was purified as only one PPIase from *M. thermolithotrophicus* (*13*). On the other hand, "molecular chaperones" like GroEL−GroES, DnaK, DnaJ, and GrpE cooperate in protein folding in the cytosol of *E. coli* (*34*, *35*). However, homologues to the genes encoding Dnak, DnaJ, and GrpE have not been found in the genomes of the hyperthermophilic archaea, *M. jannaschii* and *A. fulgidus*. Only chaperonin (type II chaperonin) genes were found (*14*, *15*), of which one from *M. thermolithotrophicus* has been characterized (*36*). *E. coli* has five FKBPs, including trigger factor, two cyclophilins, and two parvulins (*37*). It was reported that the *E. coli* FKBP homologue, trigger factor, interacts with GroEL to bind to unfolded proteins (*38*). An archaeal PPIase, MTFK, has chaperone activity and may play an important role in protein folding with chaperonin in *M. thermolithotrophicus*.

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